EXHIBIT C

RESEARCH ARTICLE

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Permitted daily exposure limits for noteworthy *N-nitrosamines*

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Abstract

A genotoxic carcinogen, *N*-nitrosodimethylamine (NDMA), was detected as a synthesis impurity in some valsartan drugs in 2018, and other *N*-nitrosamines, such as *N*-nitrosodiethylamine (NDEA), were later detected in other sartan products. N-nitrosamines are pro-mutagens that can react with DNA following metabolism to produce DNA adducts, such as O^6-alkyl-guanine. The adducts can result in DNA replication miscoding errors leading to GC>AT mutations and increased risk of genomic instability and carcinogenesis. Both NDMA and NDEA are known rodent carcinogens in male and female rats. The DNA repair enzyme, methylguanine DNA-methyltransferase can restore DNA integrity via the removal of alkyl groups from guanine in an error-free fashion and this can result in nonlinear dose responses and a point of departure or "practical threshold" for mutation at low doses of exposure. Following International recommendations (ICHM7; ICHQ3C and ICHQ3D), we calculated permissible daily exposures (PDE) for NDMA and NDEA using published rodent cancer bioassay and in vivo mutagenicity data to determine benchmark dose values and define points of departure and adjusted with appropriate uncertainty factors (UFs). PDEs for NDMA were 6.2 and 0.6 μg/person/day for cancer and mutation,

ABBREVIATIONS: AI, acceptable intake; AOP, adverse outcome pathway; API, active pharmaceutical ingredient; APROBA, approximate probabilistic analysis; BMD, benchmark dose; BMDL, benchmark dose lower bound; BMDU, benchmark dose upper bound; CES, critical effect size; CI, confidence interval; COM, Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment; CPDB, Carcinogenic Potency Database; EMA, European Medicines Agency; EMS, ethylmethanesulfonate; ENU, *N*-ethyl-*N*-nitrosourea; EPA, U.S. Environmental Protection Agency; FDA, U.S. Food and Drug Administration; HBGV, health-based guidance value; ICH, International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use; MGMT, methylguanine DNA methyltransferase; MNNG, methylnitronitrosoguanidine; MNU, *N*-methyl-*N*-nitrosourea; NDEA, *N*-nitrosodiethylamine; NDMA, *N*-nitrosodimethylamine; NOAEL, no observed adverse effect level; NOEL, no observed effect level; PDE, permissible daily exposure; PoD, point of departure; Td, threshold dose; TD_{50}, *tumorigenic dose* rate in milligrams per kilogram per day for 50% of the animals; TGR, transgenic rodent; TTC, threshold of toxicological concern; UF, uncertainty factor.

respectively, and for NDEA, 2.2 and 0.04 μg/person/day. Both PDEs are higher than the acceptable daily intake values (96 ng for NDMA and 26.5 ng for NDEA) calculated by regulatory authorities using simple linear extrapolation from carcinogenicity data. These PDE calculations using a bench-mark approach provide a more robust assessment of exposure limits compared with simple linear extrapolations and can better inform risk to patients exposed to the contaminated sartans.

1 | INTRODUCTION

N-Alkyl-nitrosamines, such as N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA), are well-studied environmental mutagens. These substances are known genotoxic carcinogens in the rat; the most comprehensive carcinogenicity dose–response data available indicates liver as the most sensitive target for tumorigenicity (Peto et al., 1991). Both nitrosamines require metabolic activation by CYP2E1, which has high inducible levels within the liver (Encell et al., 1996).

Nitrosamines are known to be metabolized to DNA reactive mutagens that result in methylation (alkylation) at the O^6 position of guanine (Arimoto-Kobayashi et al., 1997), the O^4 position of thymidine (Verna et al., 1996), and other less mutagenic lesions (Souliotis et al., 1995). When left unrepaired, the O^6-methyl-guanine can be mistakenly recognized as adenine, causing GC>AT substitution mutations during replication, and misrecognition of O^4-alkyl-thymidine can lead to TA>CG mutations. The methyl or ethyl group can be removed from O^6-alkyl-guanine or O^4-alkyl-thymidine by methylguanine DNA methyltransferase (MGMT), leaving a normal guanine and thymine residue. MGMT's ability to remove base alkylations to restore the normal wild-type DNA sequence represents an error-free DNA damage response (Christmann et al., 2011; Fahrer et al., 2015; Kaina et al., 1991; Kaina et al., 1993; Margison et al., 2003; Thomas et al., 2013) that can mechanistically account for the manifestation of a dose–response threshold. Mutagenic carcinogens often act via multiple mechanisms. NDMA and NDEA are also clastogenic along with hepatotoxic, and they can induce secondary damage via formaldehyde and reactive oxygen species production (World Health Organization, 2002). However, the most prevalent potent and cancer-related mechanism is O^6-alkyl-guanine adduction.

Dietary exposure to nitrosamines is well recognized with their presence in cured meats, tobacco smoke, and beer has been recognized for many years (Abdel-Tawab et al., 2018; EMA, 2020a; Snodin & Elder, 2019). For example, NDMA exposure from dietary consumption levels range from 0.0004 to 0.23 μg/day from cured meat, 0.0004 to 1.02 μg/day from smoked meat, and 0.0006 to 0.13 μg/day from grilled meat (FDA, 2018; Snodin & Elder, 2019). Likewise exposure via drinking water is also recognized, for example the U.S. Environmental Protection Agency (EPA) has set health reference levels for NDEA at 0.8 ng/day and NDMA at 0.6 ng/day (EMA, 2020a).

In addition to environmental exposure, NDMA was recently detected as a process-related contaminant in the pharmaceutical product valsartan. In 2018, this led to a global recall of valsartan-containing preparations in more than 22 countries, including Canada (Health Canada, 2018), Europe (EMA, 2018), and the United States (FDA, 2018). The impurity was linked to the synthetic process employed by Zhejiang Huahai Pharmaceuticals in China, a major producer of the active pharmaceutical ingredient (API) in valsartan. Certain companies specializing in producing generic pharmaceuticals purchased this API for inclusion in their products, and patients consuming the contaminated generic products were inadvertently exposed to NDMA. The U.S. Food and Drug Administration (FDA) later reported that valsartan manufactured by Hetero Labs in India also contained NDMA (Lowe, 2019). Detailed analyses of valsartan tablets revealed NDMA levels of up to 22 μg per 320-mg tablet, the highest strength available. It later became apparent that NDEA was present in the sartan drugs, irbesartan and losartan, manufactured by ScieGen and Zhejiang Huahai Pharmaceuticals, respectively (Lowe, 2019). For NDEA, 3.7 μg per 320-mg tablet has been used as a conservative worst case contamination level estimate (EMA, 2019).

The default approach for regulatory evaluation of mutagenic carcinogens, such as NDMA and NDEA, is to employ linear low-dose extrapolation from carcinogenicity dose–response data (ICH, 2017; ICH, 2018). The biology of the linear approach to risk assessment can be traced back to Muller and Stern (1940ies) as well as Knudson Jr. (1971) and others, who postulated that a small number of "hits" by mutagenic chemicals or ionizing radiation are sufficient to cause certain types of cancer. At the most conservative end of these theories, one molecule of a carcinogen is capable of causing one adduct, creating one mutation, and one mutated cancer-related gene that finally leads to cancer.

Since suitable carcinogenicity data are available, the approach recently used for risk assessment of NDMA was linear extrapolation from the *dose* (in milligrams per kilogram per day) resulting in tumor induction in 50% of the animals (i.e. TD_{50}). More specifically, in response to the valsartan contamination incident, regulatory authorities used the harmonic mean TD_{50} value for liver tumor incidence from studies in the Carcinogenic Potency Database (CPDB; 96 μg/kg/day) (Gold, 1980) and applied linear extrapolation to estimate that excess cancer risk. The values obtained indicated a theoretical excess lifetime cancer risk of 1 in 8000, assuming consumption of the highest prescribed dose of valsartan (320 mg/day), with the highest levels of NDMA, for 4 years (FDA, 2018).

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Similarly, the European Medicines Agency (EMA) stated that consumption for 7 years would be associated with a theoretical excess lifetime cancer risk of 1 in 5000. These and similar values calculated by other regulatory agencies worldwide (e.g., 1 in 11,600 (Health Canada, 2018)) led to a widespread product recall of sartan products. Similar calculations led to prohibitions of sartan products containing NDEA (EMA, 2020a).

To calculate a lifetime acceptable intake (AI) on which to base limits for these nitrosamines in pharmaceuticals, assuming a theoretical excess cancer incidence of 1 per 100,000, EMA's linear low-dose extrapolation from the TD_{50} yielded AI values of 96 ng/person/day for NDMA and 26.5 ng/person/day for NDEA (EMA, 2010). The estimates represent extremely conservative AI values for human risk assessment, as they do not consider the full character of the dose–response relationship. These AI values are also being used as interim general limits for other nitrosamine impurities that lack carcinogenicity data to calculate compound-specific limits (EMA, 2020b; FDA, 2021). Furthermore, a stricter limit of 0.03 ppm supersedes AIs, based on technical feasibility of the analytical methods (EMA, 2020a). Not all N-nitrosamines are high-potency mutagenic carcinogens, based on their harmonic-mean TD_{50} values covering. Furthermore, based on the Lhasa data, nearly 20% of nitrosamines appear to be noncarcinogenic in rodent bioassays (Thresher et al., 2020). Therefore, it could be argued that N-nitrosamine carcinogenic potency should be evaluated case by case, rather than assuming that all are of high potency based on perceptions of the Cohort of Concern.

It is now known that carcinogenesis is a multifactorial process involving mutagenic and non-mutagenic pathways and importantly the mutagenic "adverse outcome pathway" is not linear, with molecular initiating events (adducts) and key events (mutations) being repaired and/or simply not leading to a deleterious effect (Yauk et al., 2015). Furthermore, it is increasingly accepted that threshold mechanisms exist for mutagenic carcinogens (MacGregor et al., 2015a, 2015b) and an extensive analysis of carcinogens has showed that "at non-toxic doses" thresholds exist for the induction of experimental cancer for all types of carcinogen, including NDMA (Kobets & Williams, 2019).

DNA repair proficiency has been shown to have a measurable and consistent effect on the position of the PoD, through repair of low levels of specific adducts and mutations (White et al., 2020). For many nitrosamine induced adducts, MGMT is the key DNA repair enzyme, which is known to have a background level of approximately 200 molecules per cell, and is also inducible in rats (Kaina et al., 1993; Souliotis et al., 1998) but this is yet to be shown in human cells (Fritz & Kaina, 1992).

Terminology around the background levels of adducts and mutations, must be clearly defined when using mutation data for risk assessment purposes. Endogenous sources of DNA damage are defined here as including reactive oxygen species, formaldehyde, as well as sources such as gut nitrosation and cellular metabolism. Exogenous sources can be divided into two distinct categories, which are from environmental exposures including food and water, or from drug-related factors including impurities. Endogenous damage is not fully considered with the linear approach.

In addition to the biological issues, there are also several deficiencies observed when applying the linear extrapolation approach, although it is a pragmatic approach for estimating de minimis risk of mutagenic carcinogens. First, the default replicate number in a cancer bioassay is 50 animals per dose group per sex, and numbers of tumors may be small. Thus, extrapolating from the TD_{50} potency estimate to a dose theoretically associated with a cancer risk of 1 in 100,000 or 1 in 1,000,000 conflicts with the statistical power of the standard study design, and it does not consider the substantial errors associated with the estimate of the TD_{50}. Second, adjustment factors are not included in the linear extrapolation, meaning that the resulting excess cancer risk relates to the test species population and not the human population, which may be more or less susceptible than the test species. Third, confidence intervals (CIs) are not used; as the uncertainty in a cancer risk estimates may be huge; thus single value estimates of cancer risk are essentially meaningless since they do not account for innate biological variation (Slob et al., 2014).

There is an alternative to the linear low-dose extrapolation approach based on rodent carcinogenic potency (e.g., TD_{50}) based on the use of quantitative interpretation of in vivo mutagenicity dose–response data for risk assessment and regulatory decision-making (Benford, 2015; Gollapudi et al., 2013; Heflich et al., 2020; Johnson et al., 2014; Labash et al., 2015; MacGregor et al., 2015b; White et al., 2020; White & Johnson, 2016). Such an approach is particularly relevant for other impurities, where carcinogenicity data are unavailable or of poor quality, and existing in vivo mutagenicity dose–response data display a mechanistically understood response threshold (COM, 2018). More specifically, quantitative analyses of in vivo mutagenicity dose–response data can be used to determine a dose below which the likelihood of a response at a key event along the "Adverse Outcome Pathway" (AOP) in the test animal is negligible (i.e., a point of departure [PoD] such as the no observed effect level [NOEL], threshold dose [Td], or benchmark dose [BMD]). The mutation data are from somatic cells, which link to the adverse outcome of cancer, however, inclusion of germ cell mutation data, when available, would allow for assessment of heritable mutations in offspring as the adverse outcome (Cliet et al., 1993; Heflich et al., 2020).

Subsequently, the use of uncertainty factors (UFs), can be used to determine a regulatory human exposure limit, including health-based guidance values (HBGVs) such as AI or permissible daily exposure (PDE). They are also sometimes referred to as extrapolation or adjustment factors (White et al., 2020) of uncertainty or modifying factors (ICHQ3C and ICHM7 metrics such as the NOEL or BMD, which are indicative of compound potency.

With respect to quantitative dose–response analyses for the determination of mutagenic potency, several authors have highlighted advantages of the BMD approach (Hardy et al., 2017; Johnson et al., 2014; White et al., 2020; Wills et al., 2016). For example, it is essential that a PoD should have a measure of precision or uncertainty (Slob, 2014a; Slob, 2014b), and the BMD has CIs; however, the NOEL does not. Furthermore, the NOEL is heavily dependent on experimental conditions, and the BMD is to a much lesser extent (MacGregor et al., 2015a, 2015b). Johnson et al. (2014) conservatively used the lowest in vivo mutagenicity benchmark dose (BMD lower bound [BMDL]) to determine a regulatory exposure limit for the potent alkylating agents N-methyl-N-nitrosourea (MNU) and N-ethyl-N-nitrosourea (ENU), and since that time, this approach has been used in case studies and regulatory submissions and with other genotoxic

compounds (Gollapudi et al., 2020; Luijten et al., 2020; Kirkland et al., 2021).

Formal deployment of quantitative methods for interpretation of mutagenicity dose–response data was pioneered by F. Hoffmann-La Roche AG following an incident that resulted in the inadvertent patient exposure to the mutagenic alkylating agent ethylmethanesulfonate (EMS) (Gocke et al., 2009b, 2009c; Muller et al., 2009; Muller & Gocke, 2009). In the absence of carcinogenicity data, detailed analyses of transgenic rodent (TGR) mutagenicity dose–response data (using MutaMouse) were used to determine NOEL and Td values and mode-of-action mechanisms to support a threshold-based risk assessment. Their studies were used to define (a) safety factors differentiating the NOEL and the maximum human exposure level in patients who had received EMS-contaminated nelfinavir (Viracept; later shown to be a 454-fold safety margin) (Muller et al., 2009) and (b) a regulatory exposure limit below which the likelihood of a mutagenic effect was considered negligible (i.e., the PDE) (Gocke et al., 2009a; Gocke & Wall, 2009; Muller & Gocke, 2009). Application of the quantitative paradigm for regulatory interpretation of the MutaMouse dose–response data was supported by evidence for a true or practical threshold response based on error-free DNA repair by MGMT of the pro-mutagenic O^6-ethyl-guanine adducts (Muller et al., 2009). More recently, an analogous use of the PDE has been proposed to determine exposure limit values for pharmaceutical impurities, including DNA-reactive mutagens (Bercu et al., 2018). Such applications are in accordance with the following statement in the ICH M7 guideline:

> [T]he existence of mechanisms leading to a dose response that is non-linear or has a practical threshold is increasingly recognized, not only for compounds that interact with non-DNA targets but also for DNA-reactive compounds, whose effects may be modulated by, for example, rapid detoxification before coming into contact with DNA, or by effective repair of induced damage. The regulatory approach to such compounds can be based on the identification of a No-Observed Effect Level (NOEL), and use of uncertainty factors (see ICH Q3C(R5)) to calculate a PDE when data are available. (ICH, 2017)

Although regulatory authorities generally use the linear extrapolation approach to ensure the safety of a population exposed to a DNA-reactive carcinogen, its application for NDMA and NDEA does not acknowledge the fundamental scientific shift in the quantitative approaches now advocated for regulatory interpretation of mutagenicity dose–response data (Heflich et al., 2020; White et al., 2020). Furthermore, NDEA has been shown to exhibit a potential 'threshold' for carcinogenicity (Waddell et al., 2006). Indeed, the current position on nitrosamines in drug product do not utilize ICH M7 guideline options for instances when DNA repair mechanisms (i.e., those that can lead to compensatory responses at low dose exposures) are well documented (ICH, 2017). The options specified in the ICH M7(R1) guideline are to (a) carry out a linear extrapolation from the TD_{50} to calculate an AI;

(b) apply the threshold of toxicological concern (TTC) when suitable carcinogenicity data are not available; or (c) utilize available information about compensatory responses (e.g., DNA repair) to justify derivation of a PDE, the term used in ICH guidelines when nonlinear dose response is accepted (ICH, 2017). In the absence of carcinogenicity data or when such data are not sufficiently robust, the generation and analysis of in vivo mutagenicity dose–response data provides a pragmatic means for determination of a compound-specific AI/PDE. Due to the difficulty and cost of performing carcinogenicity studies, it is interesting to note that the EMA article 5(3) review (EMA, 2020b) recommends performing TGR mutation studies rather than carcinogenicity studies for N-nitrosamine impurities and using in vivo mutagenicity dose–response data to determine a BMD as the PoD for risk estimation (EMA, 2020b).

The liver was also shown to be the most sensitive tissue for induction of gene mutations in rats and mice (Akagi et al., 2015; Gollapudi et al., 1998; Jiao et al., 1997). Therefore, the rat study cancer and mutation data were selected for BMD analysis and PDE calculations enabling comparison of PDEs for cancer and mutation in the same species (Akagi et al., 2015; Gollapudi et al., 1998). We have analyzed NDMA and NDEA dose response data to determine BMDs for each nitrosamine based on existing data and applied various adjustment factors to calculate safe human exposure limits and PDEs. The analysis also provides an opportunity to compare PDE exposure limits derived from in vivo mutagenicity data with those from cancer-studies for both compounds and thereby build on the experience with the use of mutagenicity data for BMD based risk assessments. The values are subsequently evaluated via comparisons with the default TTC for non-nitrosamines as well as for known or estimated human exposures to nitrosamines via foods or therapeutic products (e.g., valsartan).

2 | MATERIALS AND METHODS

2.1 | Data selection

A literature review and analysis of the Carcinogenic Potency Database (CPDB) (Gold, 1980) now located at Lhasa (Leeds UK) (Lhasa, 2020) was carried out in order to identify the most suitable carcinogenicity data for BMD analysis. Dose spacing, total animal number, and detailed tumor analysis were considered when selecting the most relevant rodent cancer bioassay data for BMD analysis. Mutation is considered to be the most relevant key event in the AOP for cancer induced by alkylating agents, such as NDEA and NDMA, and a review of published literature was carried out to find the most suitable in vivo mutation data for both compounds. Selection criteria included the number of dose levels and replicates used since these variables are important for BMD precision.

The most extensive carcinogenicity dose–response data for NDMA and NDEA were identified in a study by Peto et al. (1991) conducted in inbred Colworth/Wistar rats. Data that has been previously used for a food-based risk assessment of nitrosamine cancer risk (Zeilmaker et al., 2010). The study included 15 doses plus vehicle controls, with 60 animals per dose group for each sex and 240 animals

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per vehicle control group. The liver was the most sensitive tissue and was defined as the most relevant tissue for the present analysis. Liver tumor data were expressed as the total numbers of malignant tumors from the different tumor types presented (Kupffer, bile duct, mesenchymal, and "liver cell"). The frequency of neoplasms observed at the lowest six doses was indistinguishable from that in the vehicle control animals (Peto et al., 1991). For our analyses, malignant cancer data (see Table 7 of Peto et al. (1991)) were used to assess the "extra risk" at the time of sampling.

TGR gene mutation data for both nitrosamines were reviewed to identify studies that had sampled liver. Although numerous studies were identified, none met OECD 488 recommendations for study design (OECD, 2011). The most suitable in vivo mutagenicity data for NDMA (4 doses) were published by Gollapudi et al. (1998) using the *LacI* gene in Big Blue rats and for NDEA (3 doses) by Akagi et al. (2015) using the *gpt* gene in rats. The studies included vehicle controls and the liver was the most sensitive tissue. For Gollapudi et al. (1998), four animals per dose were used, with daily oral gavage dosing on test days 1–5 and 8–11; animals were sacrificed on test day 12, with selected tissues collected and stored for analysis. Akagi et al. (2015) used five animals per dose across F344 and Sprague Dawley (SD) rats (NDEA was dissolved in drinking water), and animals were sacrificed at 2, 4, or 8 weeks. These studies might be repeated using the recommended study requirement of OECD 488 (OECD, 2011), which could increase the precision and acceptance of the BMD CI and associated PDE's. Other studies were rejected as inadequate, with too few dose levels (Ashby et al., 1994; Souliotis et al., 1998; Suzuki et al., 1996) or the exposure, expression, or recovery periods were too short (Butterworth et al., 1998; Jiao et al., 1997). Studies of a different mutation target, the Pig-a gene, using circulating red blood cells (Dertinger et al., 2019) were also considered; however, for these metabolically activated mutagens, mutations in liver cells of the transgenic rodents were considered the most relevant endpoint for derivation of a PDE based on mutagenicity-data.

2.2 | BMD analyses

The BMD application software (PROAST v69.2) was used to analyze and interpret the mutagenicity and carcinogenicity dose–response data for both NDEA and NDMA. The TGR mutant frequency data are continuous, and the default analyses selected were therefore the Hill and exponential models (EFSA, 2009; Hardy et al., 2017; Slob & Setzer, 2014). The combined analyses for the Akagi et al. (2015) NDEA TGR data assumed that the shape parameters (i.e., the maximum response [parameter c] and log-steepness [parameter d]) were equal for all subgroups, whereas the background response (parameter a), potency (parameter b), and within-group variation (var) were tested for subgroup dependence (Slob & Setzer, 2014; Wills et al., 2016). A critical effect size (CES) of 50% was used for the in vivo TGR mutagenicity data based on previous recommendations (Zeller et al., 2017); thus, $BMDL_{50}$ values were determined for NDEA mutagenicity data.

For the analysis of the carcinogenicity dose response data (i.e., a quantal endpoint), a CES of 10% extra risk was employed (EFSA, 2009; EPA, 2012; Hardy et al., 2017). Model averaging was used to derive the BMD and associated CI values across the suite of relevant models: two stage, log probit, probit, log logistic, logistic, gamma, exponential, Weibull, and Hill (EFSA, 2009; EPA, 2018; Hardy et al., 2017). By default in PROAST, all converged models are considered for model averaging, but for each response the user can change the set of models to be considered (EFSA, 2019). The bootstrap approach was used to examine the BMD sampling distributions and precision. The default number of bootstrap runs for calculating the BMD CIs was set to the default of 200 (Slob, 2018). Previous BMD analyses on these NDMA and NDEA cancer data, were carried out using the standard BMD approach for quantal data (EC, 2012), as model averaging was not an option at that time of analysis. Furthermore, malignant cancers were assessed here, as this was the most appropriate adverse outcome for comparison to the mutation BMD CI and PDE.

2.3 | Calculation of PDEs

PDE values were calculated according to the formula outlined in ICH M7 (ICH, 2017), with the exception that BMDL was used in place of a no observed adverse effect level (NOAEL) (Hardy et al., 2017). The lower bound of the BMDL represents a conservative estimate for the PoD calculation and this has advantages compared to the NOEL (Hardy et al., 2017). For example, a PoD should have CIs as a measure of precision and also be minimally affected by dose selection (MacGregor et al., 2015a, 2015b). PDE values were calculated according to the following equation:

$$PDE = \frac{BMDL_{50} \times 50\,kg/person}{Uncertainty\ factors} \tag{1}$$

where the default body weight was 50 kg, the value used by ICH for conservative evaluations of pharmaceutical impurities (Bercu et al., 2018).

The uncertainty/modifying factors (i.e., F1–F5, according to the nomenclature used in ICH Q3) used the following specifications ICH (2018):

- F1: species extrapolation values employed to determine the human equivalent dose. The analyses here employed a default value of 5 for rats based on standard allometric scaling factors.
- F2: interindividual variability. A maximum value of 10 was used to reflect the assumed variability in DNA repair proficiency as the major factor. Support for including a high value for F2 comes from a study comparing the BMD CI in multiple test systems, including wild-type versus repair-deficient mammalian cell test systems, with the ratio of BMD supporting the value of 10 (White et al., 2020).
- F3: exposure duration. A factor of 1 was used for the long-term study duration (over 1 year of continuous exposure in rodents
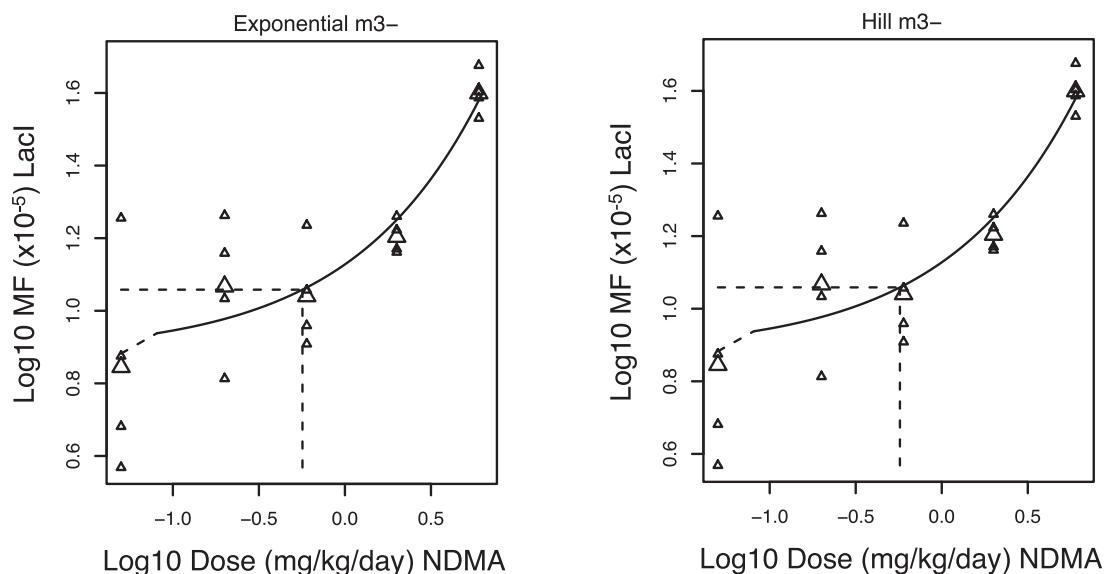
FIGURE 1   BMD analysis of in vivo transgenic gene mutation in Big Blue rats at the *LacI* locus (Gollapudi et al., 1998). PROAST v69.2 was used to assess the BMD at CES 50% using the exponential (Expon. m3-) and Hill (Hill m3-) models. The three-parameter models provided suitable fit to the data. The *x*-axes denote dose of NDMA administered and the *y*-axes show MF. The triangles represent the replicate data points. BMD, benchmark dose; CES, critical effect size; MF, mutant frequency; NDMA, *N*-nitrosodimethylamine

(Peto et al., 1991)) cancer bioassay data, and a factor of 10 was used for the TGR mutagenicity data (ICH, 2017). The TGR mutagenicity studies were 12 days for NDMA, along with 14, 28, and 56 days for NDEA. Since there is currently no accepted basis for extrapolating the duration of the mutagenicity studies, a conservative default factor of 10 was used i.e. a short-term rodent study lasting less than 3 months (ICH, 2017). Further work in this area is needed to evaluate variables such as chronic vs. acute exposures in the TGR assay.

- F4: severity of effect. Since both mutation and cancer are considered irreversible severe effects, a maximum value of 10 was used ((R7) IQC, 2018).
- F5: factor to compensate for database insufficiency (e.g., cases where a NOEL cannot be determined). This was set to 1 because the analyses are based on a BMDL values, which is considered a superior metric.

Overall, the combined UFs for both PDEs calculations were 5000 for mutagenicity and 500 for carcinogenicity, respectively. Further research to refine adjustment factor values could in time change in overall PDE value defined from the TGR data (White et al., 2020), with F2, F3, and F4 being the most open for modification based on increased understanding of biology and translation.
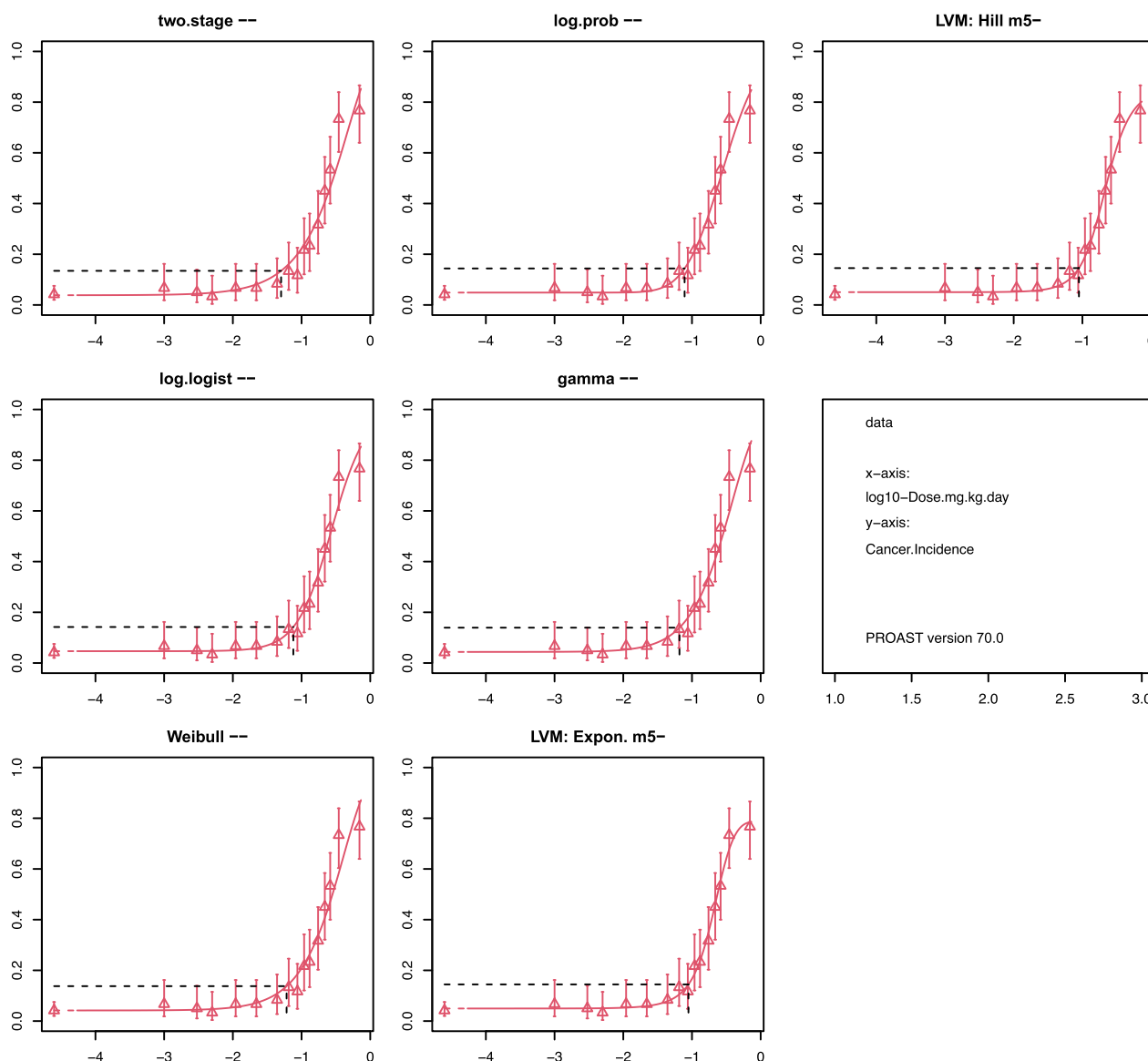
3 | RESULTS

Figure 1 shows the results of the BMD analysis for BigBlue rat *lacI* mutant frequency dose–response data (Gollapudi et al., 1998). The data were analyzed using both *lacI* and *cII* gene mutation frequencies;

analyses of the *lacI* dose–response data provided the lower, more conservative BMDL while still maintaining suitable precision (i.e., BMDL: BMD upper bound [BMDU] ratio < 100) (White et al., 2020). The analyses of the carcinogenicity dose–response data (Figure 2) yielded a very precise BMD (i.e., a ratio of the upper and lower estimates of BMD, BMDL:BMDU of <2). This same precision was also seen for the NDEA BMDL:BMDU ratios, with a higher ratio for the mutagenicity dose–response data (Figure 3) compared with the carcinogenicity dose–response data (Figure 4). The high BMDL:BMDU ratios for the NDMA TGR data, are potentially linked to the study designs being sub-optimal for dose response analysis. All BMD CIs are presented in Table 1.

PDE values calculated using carcinogenicity and mutagenicity BMD values (i.e., PDE_{cancer} and $PDE_{mutation}$, respectively) are shown in Table 1. Interestingly, the mutation PDEs are lower than those derived from carcinogenicity data. Higher sensitivity of mutation assays might be expected because mutations are predicted to occur at earlier timepoints and lower doses than cancer along the AOP, and not all mutations will progress to form tumors. However, the PDE also reflects the use of UFs that were 10 times higher in the mutation data calculations (i.e., 5000 for $PDE_{mutation}$ and 500 for PDE_{cancer}; Table 1). The difference was in the use of an F3 (treatment duration adjustment factor) of 10 for the mutation data and 1 for cancer data. The conservative nature of F3, could be considered as 1, if the 28 day study design was accepted as sufficient in order to assess mutation.

4 | DISCUSSION

Since the NDMA contamination issue in 2018, NDMA, NDEA, and the whole class of nitrosamines have received extensive regulatory

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FIGURE 2 BMD analysis of liver cancer data in Colworth/Wistar rats following exposure to NDMA (Peto et al., 1991). PROAST v70.0 was used to assess the BMD at CES 10% using the nine default quantal data models of two-stage, log-probit, probit, log-logistic, logistic, gamma, exponential, Weibull, and Hill. BMD analysis of the hepatic carcinogenicity dose–response data from table 7 of Peto et al. (1991). The dashed line is the BMD_{10}. PROAST online (https://proastweb.rivm.nl/) was used with model averaging to calculate the BMD CI bounds ($BMDL_{10} = 0.062$, 0.107 mg/kg). A complete summary of the results of the carcinogenicity BMD analyses is presented in Table S1. For completeness, analysis of the female dose–response data resulted in BMD CI bounds of $BMDL_{10} = 0.283$ and 0.367 mg/kg. The x-axes denote dose of NDMA administered and the y-axes show increased incidence of "liver cell" tumors, as a fraction of total study population. The default number of bootstrap runs (200) was used to calculate BMD CIs (EFSA, 2019). BMD, benchmark dose; BMDL, benchmark dose lower bound; CES, critical effect size; CI, confidence interval; NDMA, *N*-nitrosodimethylamine

attention. Regulatory risk assessment applied the default linear extrapolation approach from the harmonic mean TD_{50} from multiple tumorigenicity studies have resulted in AIs of 96 ng/day for NDMA and 26.5 ng/day for NDEA, respectively (EMA, 2020a; EMA, 2020b; FDA, 2021). NDMA and NDEA are the *N*-nitrosamines that are the most characterized in non-clinical safety animal models, and they are also regarded as among the more potent *N*-nitrosamine compounds based on TD_{50} values (FDA, 2021). By way of comparison, these AI values calculated using TD_{50} values, are 65-fold and 83-fold lower than the cancer PDEs calculated in the present report for NDMA and NDEA, respectively. It is also noteworthy that AI from linear extrapolation, were also calculated using $BMDL_{10}$ values at 135-215 ng/day for NDMA (EC, 2012; EMA, 2019), and 90 ng/day for NDEA (EC, 2012). The cancer AIs from TD_{50} values are 6-fold and 1.5-fold lower than the calculated mutation PDEs for NDMA and NDEA, respectively. A major contributing factor to the differences observed

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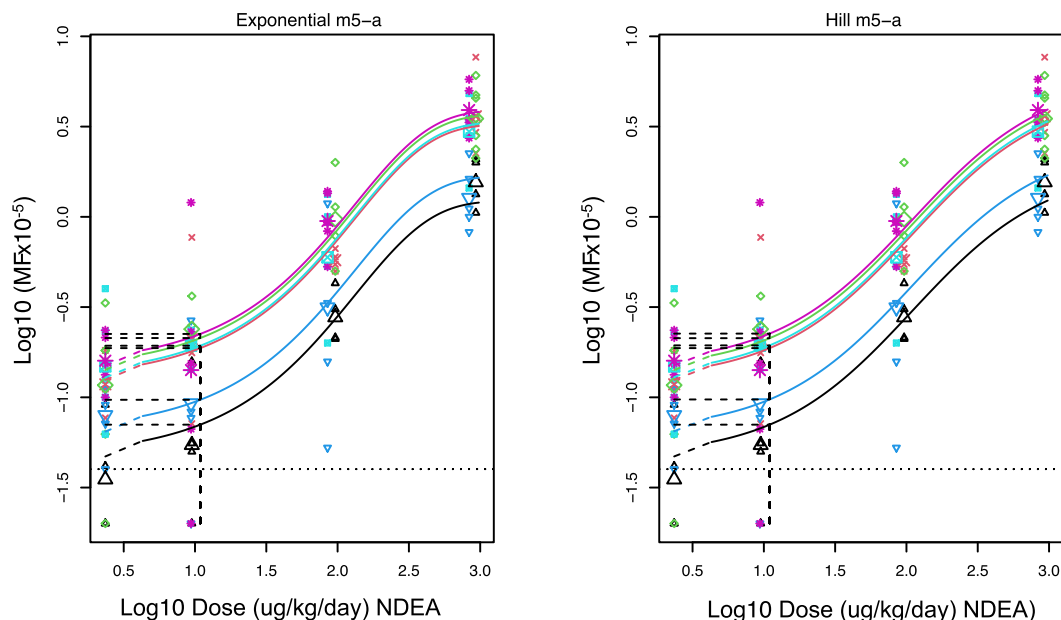
FIGURE 3 BMD covariate analysis of in vivo transgenic gene mutation in F344/gpt delta rats at the *gpt* locus using sampling time (2, 4, and 8 weeks) as the covariate (Akagi et al., 2015). PROAST v69.2 was used to assess the BMD at CES 50% using the exponential (Expon. m5-a) and Hill (Hill m5-a) models. The triangles represent the replicate data points. The different colors represent the different timepoints within 2 strains of gpt delta rats in liver; black, F344 2-weeks; blue, SD 2-weeks; red, F344 4-weeks; turquoise, SD 4-weeks; green, F344 8-weeks; SD 8-weeks. The three-parameter models provided suitable fit to the data. BMD, benchmark dose; CES, critical effect size

between the AI and PDE values, is the linear assumption and un-refined default factor of 100,000 used within the AI, compared to the non-linear assumptions, and refined adjustment factors within the PDE. Furthermore, although standard TTC values are not applicable for these nitrosamines listed in the cohort of concern (ICH, 2017), it is interesting to show that the cancer-derived PDEs for NDMA and NDEA are both higher than the default TTC value of 1.5 μg/person/day, but the mutation-derived PDEs are lower than the TTC because of the use of a high UF applied based on the short-term exposure duration of the TGR assays.
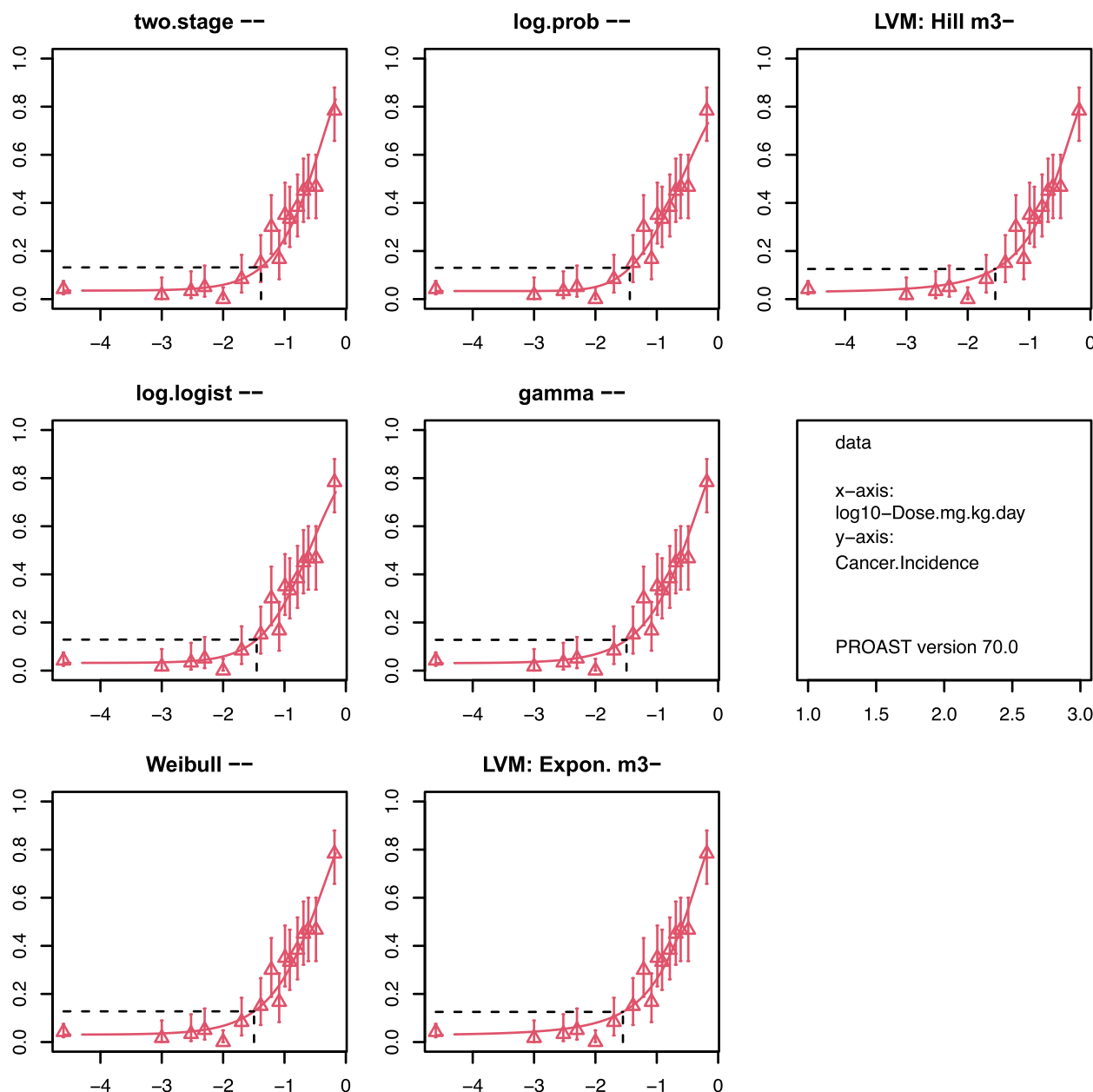
We consider that the application of the BMD should inform how *N*-nitrosamines and other DNA-reactive mutagens are assessed for regulatory purposes. In order for health authorities to support a move from the use of TD_{50} values for linear extrapolation for risk assessment toward the PDE approach for NDMA, NDEA, and other nitrosamines, there is a need for robust evidence around the underlying mechanisms by which alkylating nitrosamines cause DNA damage and cancer at low concentrations. A "threshold mechanism" with a clear PoD has been clearly presented for many alkylating agents (Muller et al., 2009; Muller & Gocke, 2009). Although chemicals such as NDMA and NDEA have quite diverse adducts spectrums, the most mutagenic and prevalent adducts are those detailed above. These DNA adducts are subject to mechanism-based thresholds linked to DNA repair, especially in the low dose region often exemplified by human exposures to impurities in pharmaceutical preparations.

The existence of mechanisms leading to a dose response that is nonlinear or have a practical threshold is increasingly recognized, not only for compounds that interact with non-DNA targets but also for DNA-reactive compounds, whose effects may be modulated by, for example, rapid detoxification before coming into contact with DNA or by effective repair of induced damage (Kobets & Williams, 2019). The regulatory approach to such compounds can be based on the identification of a NOEL and use of UFs (ICHQ3C[R6], reference 7 therein) to calculate a PDE when data are available (ICH, 2017). The impact of DNA repair on the potency of DNA-reactive mutagens is discussed in White et al. (2020), in which the BMDL was lower when DNA repair was knocked down across every study investigated. Additional studies using a mammalian gene mutation endpoint along with DNA repair knockdown would support the impact of DNA repair on the potency of NDMA and NDEA, as previously shown using a bacterial gene mutation endpoint (Arimoto-Kobayashi et al., 1997). There are extensive data for this on potent mutagens with similar adduct and mutation profiles in mammalian cells, including methylnitronitrosoguanidine (MNNG) and MNU (Beranek, 1990; Jenkins et al., 2005; Kaina et al., 1991).

For alkylating nitrosamines, gene mutation is the type of genetic damage most linked to cancer. Therefore, in the absence of cancer data, in vivo mutagenicity data would be the preferred endpoint for calculation of a HBGV (EMA, 2020b; EMA, 2020c).

With respect to the levels of NDMA in valsartan tablets (which varied to levels ≤20 μg/day), based on the aforementioned BMD and PDE modeling approach, the calculated mutagenicity PDE alone would indicate that certain batches of 320-mg valsartan tablets that contained levels of NDMA that would not represent a theoretical mutagenicity risk and hence an increased theoretical excess

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FIGURE 4   BMD analysis of liver cancer data in Colworth/Wistar rats following exposure to NDEA (Peto et al., 1991) . PROAST v70.0 was used to assess the BMD at CES 10% using the nine default quantal data models of two-stage, log-probit, probit, log-logistic, logistic, gamma, exponential, Weibull, and Hill. BMD analysis of the hepatic carcinogenicity dose–response data from table 7 of Peto et al. (1991). PROAST online (https://proastweb.rivm.nl/) was used with model averaging to calculate the BMD CI bounds (BMDL$_{10}$ = 0.022, 0.046 mg/kg). The dashed line is the BMD$_{10}$. A complete summary of the results of the carcinogenicity BMD analyses is presented in Table S2. For completeness, analysis of the female dose–response data resulted in BMD CI bounds of BMDL$_{10}$ = 0.023 and 0.041 mg/kg. The x-axes denote dose of NDEA administered and the y-axes show increased incidence of "liver cell" tumors, as a fraction of total study population. The default number of bootstrap runs (200) was used for calculating BMD CIs (EFSA, 2019). BMD, benchmark dose; BMDL, benchmark dose lower bound; CES, critical effect size; CI, confidence interval; NDEA, N-nitrosodiethylamine

cancer risk in the human population. Likewise, the calculated carcinogenicity PDE would also indicate that numerous batches of 320-mg valsartan tablets contain levels of NDMA that would not represent an increase in the theoretical excess cancer risk in the human population.

An outcome of this study is a potential framework for how mutation data can be used for human health risk assessments, with a focus on potent mutagenic impurities. Progress has been made in using the BMD approach on mutagenicity data to calculate PoD (Gollapudi et al., 2013; Johnson et al., 2014; MacGregor et al., 2015a), and also

TABLE 1   PDE values calculated using mutagenicity and carcinogenicity dose–response data for NDMA and NDEA

Parameter	NDMA		NDEA	
	$PDE_{mutation}$	PDE_{cancer}	$PDE_{mutation}$	PDE_{cancer}
Endpoint modeled	Increase in *lacI* MF for Big Blue rats, liver	Increased incidence of liver cell tumors in Colworth rats	Increase in *gpt* MF for liver in F344 and SD/gpt delta rats	Increased incidence of liver cell tumors in Colworth rats
Data source	Gollapudi et al. (1998)	Peto et al. (1991)	Akagi et al. (2015)	Peto et al. (1991)
CES (% increase over mean controls)	50	10	50	10
BMD (mg/kg/day)	0.37	0.088	0.011	0.035
BMDU (mg/kg/day)	2.34	0.107	0.028	0.046
BMDL (mg/kg/day)	0.06	0.062	0.004	0.022
Composite UF	5000	500	5000	500
PDE (µg/person/day)	0.6	6.2	0.04	2.2

Note: Equation 1 was used for PDE calculations. BMD, benchmark dose; BMDL, benchmark dose lower bound; BMDU, benchmark dose upper bound; CES, critical effect size; MF, mutant frequency $\times 10^{-5}$; NDEA, *N*-nitrosodiethylamine; NDMA, *N*-nitrosodimethylamine; PDE, permissible daily exposure; UF, uncertainty factor.

around the understanding that mutation is a relevant endpoint for human health risk assessment (Heflich et al., 2020). However, further work is needed to solidify the guidance, particularly on selection of the adjustment factors (White et al., 2020). Approaches such as the approximate probabilistic analysis (APROBA) can be used to account for uncertainty in the HBGV (EPA, 2000; International Programme on Chemical Safety, 2014; White et al., 2020), and their application to mutagenicity data is of major interest in developing the use of these data for protecting the human population from additional cancer risk. The PDEs derived from the mutagenicity dose–response data (Table 1) were determined using a conservative composite UF of 5000. There is currently no agreed basis for selection of appropriate UF values for interpretation of in vivo genetic toxicity dose–response data. White et al. (2020) show a suggested approach for defining these factors with some potential to reduce the values of the UFs used in this report. Through this suggested approach, those in the population with conditions such as DNA repair deficiency are protected. This work is being expanded upon, to refine and solidify guidance for recommendations of factors for use within genetic toxicity-based risk assessments. Our current approach uses the most conservative factors, leaving future opportunities to refine the values for use in calculating genetic toxicity driven PDEs.

Detailed considerations of options for each factor are beyond the scope of this work, although international expert groups, including the Health and Environmental Sciences Institute Genetic Toxicology Technical Committee and the 2021 International Workshop on Genotoxicity Testing, intend to address this task and provide further guidance in due course.

CONFLICT OF INTEREST
G.E.J. is a consultant who evaluates the risks posed by pharmaceutical impurities. His clients did not influence the content of this manuscript. The other authors do not declare any conflicts of interest.

AUTHOR CONTRIBUTIONS
George E. Johnson designed the study and analyzed the data and prepared draft figures and tables. George E. Johnson prepared the manuscript draft with important intellectual input from all authors. All authors approved the final manuscript.

DATA AVAILABILITY STATEMENT
The data used in these analyses are available from the cited publications.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.